CERTIFICATE OF VERIFICATION

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state that the attached document is true and complete translation to the best of my knowledge of Japanese Patent Application No. 054977/1995.

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Signature: Seiya Fujino

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[Title of the Invention] Novel proteins and methods for producing the proteins

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【File name】 SPECIFICATION

【Title of the Invention】

NOVEL PROTEINS AND METHODS FOR PRODUCING THE PROTEINS

【Claims】

【Claim 1】 A protein characterized by the following physicochemical properties and having biological activity to inhibit osteoclast differentiation and/or maturation.

(a) molecular weights on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions

(b) affinity; affinity to cation-exchanger, Cibacron blue gel and heparin.

(c) thermostability

; its biological activity to inhibit osteoclast differentiation and/or maturation is decreased by heating at 70℃ for 10 min. or at 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

(d) amino acid sequence

; internal amino acid sequences provided in sequence Nos. 1 and 2.

【Claim 2】 The protein according to claim 1 characterized in produced by human fibroblasts.

【Claim 3】 A method of producing the protein according to claim 1 or 2 characterized in by cultivating human fibroblast cells, purifying the cultured solution by adsorption and elution in ion-exchange column, heparin-column, Cibacron blue-column, and reversed phase-column chromatography.

【Claim 4】 A method of producing the protein according to Claim 3 by cultivating human fibroblast cells on a carrier of alumina ceramic pieces.

【Detailed Explanation of the Invention】

【0001】

【Field of the Invention】

This invention relates to a novel protein exhibiting inhibitory activity on differentiation and/or maturation of osteoclast, that is osteoclast inhibitory factor (OCIF), and methods for producing the protein.

【0002】

【Description of the Prior Arts】

Human bones are always remodeling by the repeated process of resorption and reconstitution. In the process, osteoblasts and osteoclasts are considered to be the cells mainly responsible for bone formation and bone resorption, respectively. A typical example of disease caused by the abnormal

progress of bone metabolism includes osteoporosis. The disease is known to be provoked by the condition in which bone resorption by osteoclasts exceeds bone formation by osteoblasts, but the mechanism of osteoporosis has not yet been completely elucidated. Osteoporosis causes pain in the bone and makes the bone fragile, leading to fracture. Since osteoporosis increases the number of bedridden old people, it has become a social issue with the increasing number of old people. Therefore, development of efficacious drugs for the treatment of disease are eagerly expected to be developed. Bone mass reduction caused by the abnormal bone metabolism is thought to be prevented by inhibiting bone resorption, improving bone formation, or improving the balanced metabolism.

[0003]

Bone formation is expected to be promoted by stimulating growth, differentiation, or activation of osteoblasts, or inhibition of growth, differentiation or activation of osteoclast. Many physiologically active proteins including cytokines stimulating the growth of osteoblasts are focused attention and active investigation have been carried out and reported, e.g. fibroblast growth factor (FGF) [Rodan, S.B. *et al.*, Endocrinology, *121*, 1917 (1987)], insulin-like growth factor-I (IGF-I) [Hook, J.M. *et al.*, Endocrinology, *122*, 254 (1988)], insulin-like growth factor-II (IGF-II)

[McCarthy, T. *et al.*, Endocrinology, *124*, 301 (1989)], activin A [Centrella, M., *et al.* Mol. Cell. Biol., *11*, 250 (1991)], transforming growth factor-β [Noda M., The Bone, *2*, 29 (1988)], vasculotropin [Varonique, M., *et al.*, Biochem. Biophys. Res. Comm., *199*, 380 (1994)] and bone morphogenetic protein (BMP) BMP-2 [Yamaguchi, A., *et al.*, J. Cell Biol., *113*, 682 (1991), OP-1; [Sampath, T.K. *et al.*, J. Biol. Chem., *267*, 20532 (1992), Kuntsen, R., *et al.*, Biochem. Biophys. Res. Commun., *194*, 1352 (1993) have been reported.

【0004】

On the other hand, cytokines which inhibits differentiation and/or maturation of osteoclast such as transforming growth factor-β [Chenu, C. *et al.*, Proc. Natl. Acad. Sci., U.S.A. *85*, 5683 (1983)] and interleukin-4 [Kasano, K. *et al.* Bone Miner., *21*, 179 (1993)] have been reported, and calcitonin [Bone-Miner., *17*, 347 (1992)] macrophage colony-stimulating factor [Hattersley, G., *et al.* J. Cell. Physiol., *137*, 199 (1988)], interleukin-4 [Watanabe, K., *et al.* Biochem. Biophys. Res. Commun., *172*, 1035 (1990)], and interferon-γ [Gowen, M. *et al.*, J. Bone Miner. Res., *1*, 469 (1986)] have been reported to inhibit the resorption of bone by osteoclast.

【0005】

These cytokines are expected to be efficacious drugs for

improving bone mass reduction by stimulating bone formation and/or by inhibiting bone resorption. Some cytokines such as insulin like growth factor-I and parosteosis factors are now investigated in clinical trials. Calcitonin is already used as a drug to care osteoporosis and diminishes pain in osteoporosis.

【0006】

【Problems to be solved by the Invention】

Examples of drug products now clinically utilized for the treatment of bone diseases and for shortening the treatment period are active vitamin D_3, calcitonin and its derivatives, hormones such as estradiol, ipriflavon and calcium preparations. However, these drug products do not provide satisfactory therapeutic effects, and novel drug substances have been expected to be developed. As mentioned above, bone metabolism is controlled in the balance between bone resorption and bone formation. Therefore, cytokines which inhibits osteoclast differentiation and/or maturation are expected to be developed as drugs for the treatment of bone diseases such as osteoporosis. Therefore, the object of the present invention is to provide a novel osteoclastogenesis inhibitory factor and an effective process for the production thereof.

【0007】

【Method to solve the Problems】

The inventors have intensively searched for osteoclastogenesis inhibitory factors and have found a novel osteoclastogenesis inhibitory factor (OCIF) which inhibits differentiation and/or maturation of osteoclasts in human embryonic fibroblast IMR-90 (ATCC CCL186) conditioned medium.

The inventors have established a method for accumulating the protein in a high concentration by culturing IMR-90 cells using alumina ceramic pieces as cell matrices.

The inventors have also established an efficient method for isolating and purifying the protein, OCIF, from the IMR-90 conditioned medium using sequential column chromatographies, including ion-exchange, heparin, Cibacron-blue, and reversed phase.

【0008】

This invention relates to a protein derived from human fetal lung fibroblast cells and characterized by the following properties.

(a) molecular weight on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions.

(b) high affinity to cation-exchange column, Cibacron blue column, and heparin column.

(c) biological activity to inhibit osteoclast differentiation and/or maturation

; its activity is decreased by heating at 70℃ for 10 min. or 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

The structure of the OCIF protein is different from the previously reported proteins that inhibit osteoclast formation.

The invention includes a method for obtaining isolated and purified OCIF protein, comprising; (1) culturing human fibroblasts, (2) applying the cultured medium to a heparin column to obtain the adsorbed fraction, (3) treating the eluate with anion exchange column to give non-adsorbed fraction, (4) purifying the OCIF protein using cation-exchange column, (5) purifying the OCIF protein eluate using heparin, Cibacron blue and reversed-phase columns.

The column treatment of the present invention provides similar efficacious purification of OCIF protein not only in treatment of cultured mixtures with heparin Sepharose column but also in batch treatment of cultured mixture with heparin Sepharose. The Cibacron blue column is prepared by combining Cibacron blue F3GA on a carrier of Sepharose (cellulose) and generally called blue column.

Furthermore, the present invention relates to an effective process for the production of aforementioned protein

using cell culture on an alumina ceramic carrier as a culture medium.

[0009]

The OCIF protein of the present invention can be isolated from human fibroblast conditioned medium with high yield. Isolation procedure of OCIF is based on ordinary techniques for purifying proteins from biomaterials, in accordance with physical and chemical properties of the OCIF protein. For example, concentration procedure includes ordinary biochemical techniques such as ultrafiltration, lyophilization, and salting out. Purification procedure includes combinations of several chromatographic techniques for purifying proteins such as ion-exchange chromatography, affinity chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, and preparative gel electrophoresis. Human fibroblast for production of OCIF protein, preferably IMR-90 (ATCC CCL 186) is used. A method for producing the IMR-90 conditioned medium is preferably a process comprising, adhering human fetal lung fibroblast IMR-90 cells to alumina ceramic pieces in roller-bottles, using DMEM medium supplemented with 5% new born calf serum for the cell culture, and cultivating the cells in roller-bottles for seven to 10 days without rotation. 0.1% CHAPS (3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate, Sigma) is preferably added to the buffer as a detergent in

the purification steps of OCIF protein.

【0010】

The purification of protein OCIF of the present invention is carried out as follows: the cultured solution is charged to a heparin column (heparin Sepharose CL-6B, Pharmacia) and eluted with 10 mM Tris-HCl buffer containing 2M NaCl at pH 7.5 to give heparin adsorptive OCIF fraction. The fraction is charged to Q/anion exchange column (HiLoad-Q/FF. Pharmacia) and the non-adsorbed fraction are collected to give heparin adsorptive basic OCIF fraction. The obtained OCIF active fraction is successively treated with S/cation exchange column (HiLoad-S/Hp, Pharmacia), heparin column (heparin-5PW, Tosoh), Cibacron blue column (blue-5PW, Tosoh), and reversed phase column (BU-300C4, Applied Corp.) for isolation and purification. The prepared fraction can be defined by the aforementioned characteristic features. The OCIF activity was determined according to the method of Kumegawa, M. *et al.*, [Protein, Nucleic acid, Enzyme, *34*, 999 (1989)] and Takahashi, N. *et al.* [Endocrinology, *122*, 1373 (1988)]. That is, about 17-day-old mouse bone marrow cells are used as target cells and the inhibitory activity of osteoclast cells in the presence of active vitamin D_3 (calcitriol) is determined by the inhibition of induction of tartaric acid resistant acid phosphatase activity.

【0011】

The OCIF protein of the invention is useful as a pharmaceutical ingredients for treating or improving decreased bone mass such as osteoporosis, decreases of bone mass such as abnormal bone metabolism. The OCIF protein is also useful as an antigen to establish immunological diagnosis of the diseases. Pharmaceutical preparations containing the OCIF protein as an active ingredients are formulated and can be orally or parenterally administered. The preparation contains the OCIF protein of the present invention as an efficacious ingredient and is safely administered to human.

Examples of the pharmaceutical preparations include compositions for injection, intravenous drip infusion, suppositories, nasal preparations, sublingual preparations, and for percutaneous absorption. The preparation for injection is a mixture of the OCIF protein with pharmacologically efficacious amount and pharmaceutically-acceptable carriers. The carriers are vehicles and/or activators which are generally added to ingredients for injection, e.g. amino acids, saccharides, cellulose derivatives and other organic and inorganic compounds. When the OCIF protein is mixed with the vehicles and/or activators to prepare injections, pH adjuster, buffer, stabilizer, solubilizing agent, etc. can be added if necessary.

【0012】

【EXAMPLES】

The present invention will be further explained by providing examples, however, the scopes of the invention are not restricted by these examples.

Preparation of a conditioned medium of human fibroblast IMR-90

Human fetal lung fibroblast IMR-90 (ATCC-CCL 186) cells were cultured on alumina ceramic pieces (80 g) (alumina: 99.5%, manufactured by Toshiba Ceramic K.K.) in DMEM medium (manufactured by Gibco BRL Co.) supplemented with 5% FCS and 10 mM HEPES buffer (500 ml/roller bottle) at 37℃ in the presence of 5% CO_2 for 7-10 days using 60 roller bottles (490 cm^2, 110 x 171 mm, manufactured by Coning Co.) without rotating the bottles. The conditioned medium was harvested, and a fresh medium was added to the roller bottles. About 30 L of IMR-90 conditioned medium per batch culture was obtained. The conditioned medium was designated as sample 1.

【0013】

Assay method for osteoclast development inhibitory activity

Osteoclast development inhibitory activity of the present invention was assayed by measuring tartrate-resistant acid phosphatase (TRAP) activity according to the methods of M. Kumegawa *et al.* [Protein, Nucleic Acid, Enzyme, 34, 999

(1989)] and N. Takahashi *et al.* [Endocrinology, *122*, 1373 (1988)]. Briefly, in 96-well microtiter plate, 100 µl of the sample diluted with α-MEM (GIBCO BRL Co.) containing 10% FBS and 2 x 10^{-8} of active vitamin D_3 and 100 µl of a suspension of 3 x 10^5 bone marrow cells obtained from 17-days-old mice in a α-MEM containing 10% FBS was inoculated and cultured for seven days at 37°C in humidified 5% CO_2. During incubation 160 µl of old medium in each well was replaced with 160 µl of the sample diluted in α-MEM containing 1 x 10^{-8} M active vitamin D_3 and 10% FBS on days three and five after the inoculation of cells. On day seven, after washing with phosphate buffered saline, cells were fixed with ethanol/acetone (1:1) for one min. at room temperature, and then osteoclast development was tested for acid phosphatase activity using a kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease of TRAP positive cells was taken as an indication of an OCIF activity.

【0014】

Purification of OCIF

i) Purification on a column of heparin Sepharose CL-6B

The IMR-90 conditioned medium (ca. 90 L) (sample 1) was filtered with 0.22 µm membrane filter (hydrophilic Millidisk, 2,000 cm^2, manufactured by Milipore Co.), and was divided into three parts. Each part (30 L) was applied to a

heparin Sepharose CL-6B column (5 x 4.1 cm) equilibrated with 10 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5. After washing the column with 10 mM Tris-HCl buffer, pH 7.5 at a flow rate of 500 ml/hr., heparin Sepharose CL-6B adsorbent protein fraction (900 ml) was eluted with 10 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl. This fraction was designated as sample 2.

【0015】

ii) Purification on a column of HiLoad-Q/FF

The heparin Sepharose adsorbent fraction (sample 2) was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, supplemented with CHAPS to a final concentration of 0.1%, incubated at 4℃ overnight, and divided into two parts. Each part was then applied to an anion-exchange column (HiLoad-Q/FF, 2.6 x 10 cm, manufactured by Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5 to obtain a non-adsorbed fraction (1,000 ml). This fraction was designated as sample 3.

【0016】

iii) Purification on a column of HiLoad-S/HP

The HiLoad-Q non-adsorbent fraction (sample 3) was applied to a cation-exchange column (HiLoad-S/HP, 2.6 x 10 cm, manufactured by Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5. After washing the column with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5,

the adsorbed protein was eluted with a linear gradient of 0-1 M NaCl over 100 min. at a flow rate of eight ml/min. and fractions of each 12 ml volume were collected. Each 10 fraction of Nos. 1-40 was composed to form one portion. Each 100 μl of the four portions was tested for OCIF activity. OCIF activity was observed in fractions from 11-30 as shown in Figure 1. The fractions from 21-30 having higher specific activity were collected and designated as sample 4.

[0017]

iv) Purification on an affinity column of heparin-5PW (heparin-5PW)

One hundred and twenty ml of HiLoad-S fractions of Nos. 21-30 (sample 4) was diluted with 240 ml of 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5, and applied to an affinity column of heparin-5PW (0.8 x 7.5 cm, manufactured by Tosoh Corp.) which was equilibrated with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5. The adsorbed protein was washed with 50mM Tris-HCl, 0.1% CHAPS,pH 7.5, and eluted with a linear gradient of 0-2 M NaCl over 60 min. at a flow rate of 0.5 ml/min. and fractions of 0.5 ml each volume were collected. In each fraction, 50 μl, was tested for OCIF activity. A fraction (10 ml) eluted with NaCl concentration from 0.7-1.3 M NaCl was found to have OCIF activity and was designated as sample 5.

【0018】

v) Purification on an affinity column of blue 5PW (blue-5PW)

Ten ml of sample 5 was diluted with 190 ml of 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5 and applied to an affinity column of blue-5PW (0.8 x 7.5 cm, manufactured by Tosoh Corp.) which was equilibrated with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5. After washing the column with 50 mM Tris-HCl buffer, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient of 0-2 M NaCl over 60 min. at a flow rate of 0.5 ml/min., and fractions of 0.5 ml each volume were collected. Using 25 μl of the each fraction, OCIF activity was evaluated. Fraction Nos. 49-70 eluted with about 1.0-1.6 M NaCl was found to exhibit OCIF activity.

【0019】

vi) Purification on a reversed phase column

One ml of the blue 5PW fraction obtained by collecting fraction Nos. 49-50 was acidified with 10 μl of 25% TFA and applied to a reversed phase C4 column (BU-300, 2.1 x 220 mm, manufactured by Perkin-Elmer) which was equilibrated with 0.1% of TFA and 25% of acetonitrile. The adsorbed protein was eluted with a linear gradient of up to 55% acetonitrile over 60 min. at a flow rate of 0.2 ml/min., and each protein peak was collected (Fig. 3). One hundred μl of the each peak fraction was tested for OCIF activity. The peaks 6-7

were found to have concentration dependent OCIF activity. The results are shown in Table 1.

【0020】

【Table 1】

OCIF activity eluted from reversed phase column

Sample	Dilution rate			
	1/40	1/120	1/360	1/1080
peak 13	++	++	+	-
peak 14	++	+	-	-

[++: OCIF activity inhibiting osteoclast development more than 80%.
+: OCIF activity inhibiting osteoclast development between 30% and 80%, and
-: no OCIF activity.]

【0021】

Molecular weight determination of OCIF protein

Each 40 µl of two protein peaks (6 and 7) with OCIF activity was subjected to SDS-PAGE under reducing and non-reducing conditions. Each 20 µl peak fraction was collected in two tubes concentrated by centrifugation and dissolved in 1.5 µl of a mixture of 10 mM Tris-HCl buffer, pH 8, one mM EDTA, 2.5% SDS and 0.01% bromophenol blue, and incubated at 37℃ overnight (with or without 5% of 2-mercaptoethanol). Each 1.0 µl of sample was then analyzed by SDS-PAGE with a gradient gel of 10-15% acrylamide (manufactured by Pharmacia

Co.) and an electrophoresis device (Phast System, manufactured by Pharmacia Co.). The following molecular weight marker proteins were used to calculate molecular weight: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD). After electrophoresis, protein bands were visualized by silver stain with Phast Gel Silver Stain kit (Pharmacia Co.). The results are shown in Fig. 4.

【0022】

A protein band with about 60 kD was detected in the peak 6 under both reducing and non-reducing conditions. A protein band with about 60 kD was detected under reducing conditions and a protein band with about 120 kD was detected under non-reducing conditions in the peak 7. Therefore, the protein in the peak 7 was considered to be a homodimer of the protein in the peak 6.

【0023】

Thermostability test of OCIF

Twenty μl of sample from the blue-5PW fractions Nos. 51-52 was incubated for 10 min. at 70℃ or 90℃, or for 30 min. at 56℃. These treated samples were tested for OCIF activity according to the aforementioned method. The results are shown in Table 2.

【0024】

【Table 2】

Heat stability of OCIF

Sample	Dilution rate		
	1/300	1/900	1/2700
untreated	++	+	-
70℃, 10 min.	+	-	-
56℃, 30 min.	+	-	-
90℃, 10 min.	-	-	-

[++: OCIF activity inhibiting osteoclast development more than 80%.
+: OCIF activity inhibiting osteoclast development between 30% and 80%, and
-: no OCIF activity.]

【0025】

(5) Determination of internal amino acid sequence of OCIF protein

The each of composed two fraction Nos. 51-70 obtained from blue-5PW column were combined to one ml, and each one ml of samples was acidified with 10 μl of 25% TFA, and was applied to a reversed phase C4 column (BU-300, 2.1 x 220 mm, manufactured by Perkin-Elmer Corp.) which was equilibrated with 0.1% of TFA and 25% of acetonitrile. The adsorbed protein was eluted with a linear gradient of up to 55%

acetonitrile over 60 min. at a flow rate of 0.2 ml/min., and the protein fractions corresponding to peaks 6 and 7 were collected, respectively. The protein in each peak was applied to a protein sequencer (PROCISE 494, manufactured by Applied Corp.). However, the N-terminal sequence of the protein in each peak could not be analyzed. Therefore, N-terminal of the protein in each peak might be blocked. So, internal amino acid sequences of these proteins were analyzed. The protein in peak 6 or 7 was concentrated by centrifugation and 50 μl of 0.5 M Tris-HCl buffer, pH 8.5, containing 100 μg of dithiothreitol, 10 mM EDTA, 7 M guanidine-HCl and 1% CHAPS was added to the each sample protein, and the mixture was allowed to stand for reduction for four hrs. at room temperature and incubated overnight with 0.2 μl of 4-vinylpyridine in the dark at a room temperature. The each sample which was mixed with 1 μl of 25% TFA was applied to reversed phase C4 column (BU-300, 2.1 x 30 mm, manufactured by Applied) equilibrated with 0.1% TFA and 20% acetonitrile. The pyridylethylated OCIF protein was eluted with a linear gradient of up to 50% acetonitrile over 30 min. at a flow rate of 0.3 ml/min., and each protein peak was collected. The pyridylethylated protein was concentrated and dissolved in 25 μl of 0.1 M Tris-HCl buffer, pH 9,

containing eight M urea and 0.1% Tween 80. Seventy three μl of 0.1 M Tris-HCl buffer, pH 9, and 0.02 μg of API (lysyl endopeptidase, Wako Pure Chemical Ltd.) were added to the sample, and incubated at 37℃ for 15 hrs. The sample which was acidified with 1 μl of 25% TFA was applied to a reversed phase C8 column (RP-300, 2.1 x 220 mm, manufactured by Perkin-Elmer Corp.) which was equilibrated with 0.1% TFA. The peptide fragments were eluted from the column with a linear gradient up to 50% acetonitrile over 70 min. at a flow rate of 0.2 ml/min., and each peptide (Fig.5) was collected. The amino acid sequence of each peptide fragment (P1-P2) was analyzede with a protein sequencer. The sequences of the peptides were shown in the Sequence Nos. 1-2, respectively.

【0026】

【Effect of the Present Invention】

A novel protein having inhibitory activity for osteoclast formation and a method of effective production of the protein are provided by the present invention. The protein of the present invention has inhibitory activity on osteoclast formation and is useful for the treatment of various diseases such as bone mass reduction including osteoporosis and immunological diagnosis thereof.

【0027】

【Sequence table】

Sequence No: 1

Length of sequence: 6

Type of sequence: amino acids

No. of chain: 1

Topology: linear

Molecular type: peptide (internal amino acid of protein)

Sequence: Xaa-Tyr-His-Phe-Pro-Lys
1 5

【0028】

Sequence No: 2

Length of sequence: 14

Type of sequence: amino acids

No. of chain: 1

Topology: linear

Molecular type: peptide (internal amino acid of protein)

Sequence: Xaa-Gln-His-Ser-Xaa-Gln-Glu-Gln-Thr-Phe-Gln-Leu-
1 5 10
Xaa-Lys

【Brief description of the drawings】

【Fig. 1】

Figure 1 shows the elution pattern of crude OCIF protein (Hiload-Q/FF through fraction ; sample 3) from Hiload-S/HP column.

【Fig. 2】

Figure 2 shows the elution pattern of crude OCIF protein (heparin-5PW fraction ; sample 5) from blue-5PW column.

【Fig. 3】

Figure 3 shows the elution pattern of OCIF protein (blue-5PW fractions 49 to 50) from reversed-phase column.

【Fig. 4】

Figure 4 shows the SDS-PAGE of isolated OCIF proteins under reducing conditions or non-reducing conditions.

【Description of the lanes】

lanes 1,4 ; molecular weight marker proteins

lanes 2,5 ; OCIF protein of peak 6

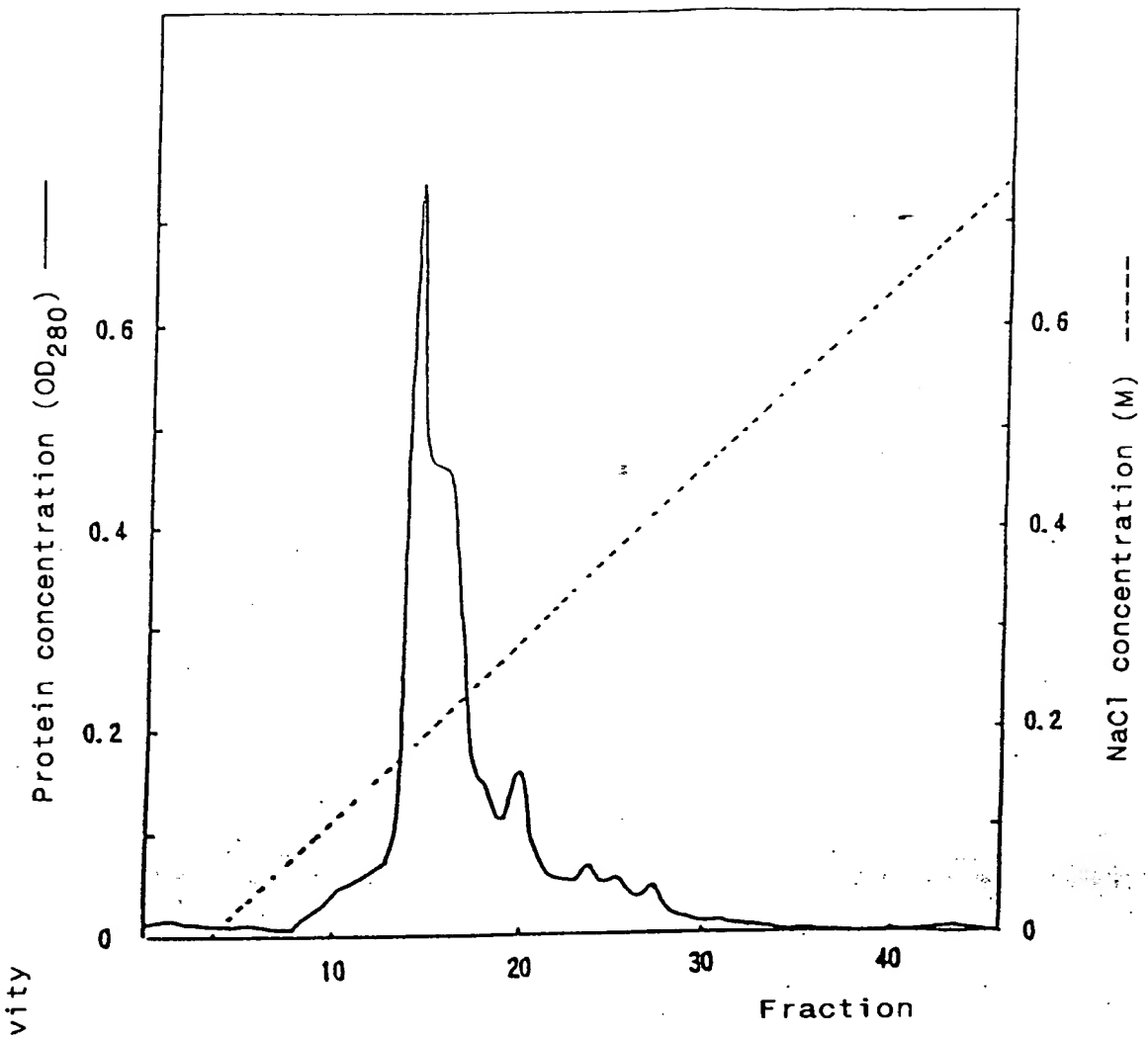
lanes 3,6 ; OCIF protein of peak 7

【Fig. 5】

Figure 5 shows the elution pattern of OCIF protein of peak 7 from OCIF protein after pyridylethylation under reducing condition followed by digestion with lysylendopeptidase on a reversed-phase column.

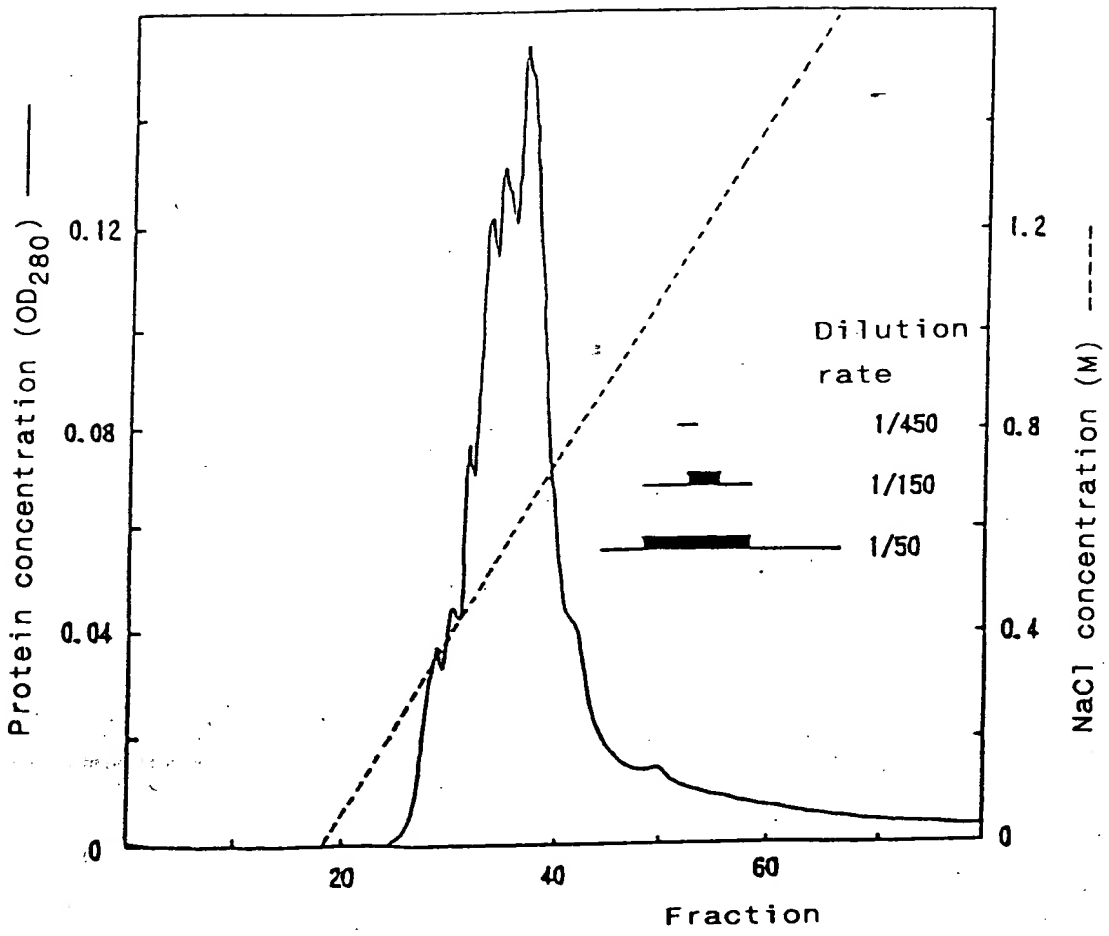
[File name] Drawing

[Figure 1]



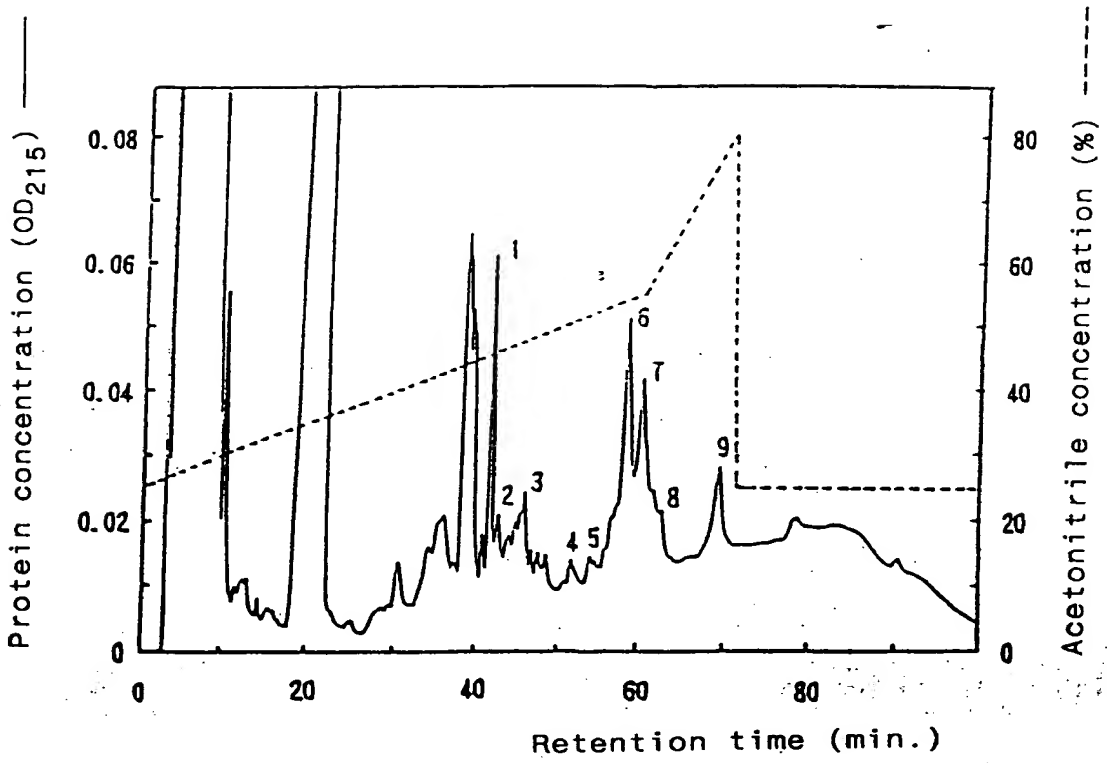
	Dilution rate			
OCIF activity	1/100	–	++	++	–
	1/500	–	++	++	–

[Figure 2]

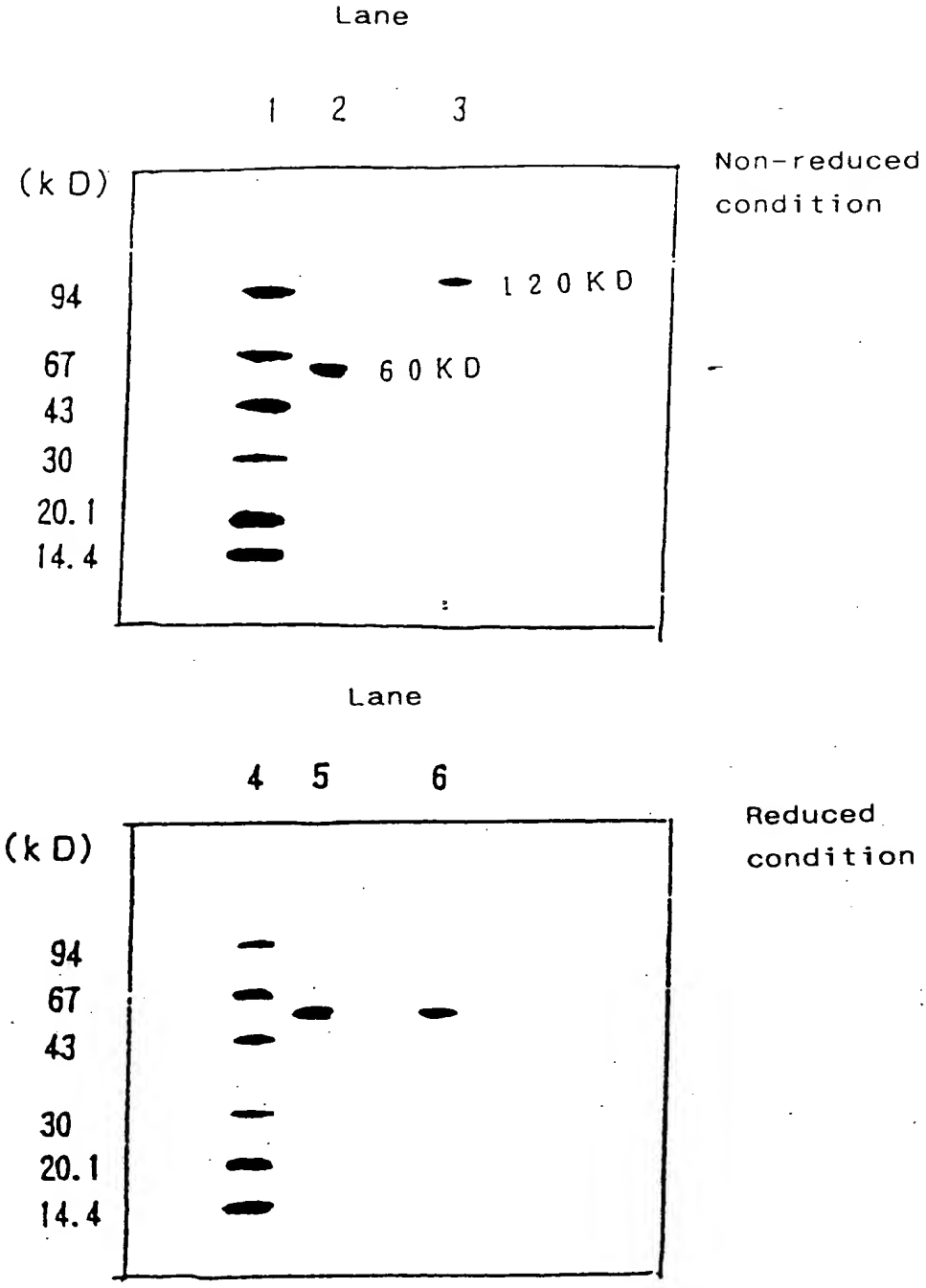


OCIF activity +: ——— , ++: ■■■■

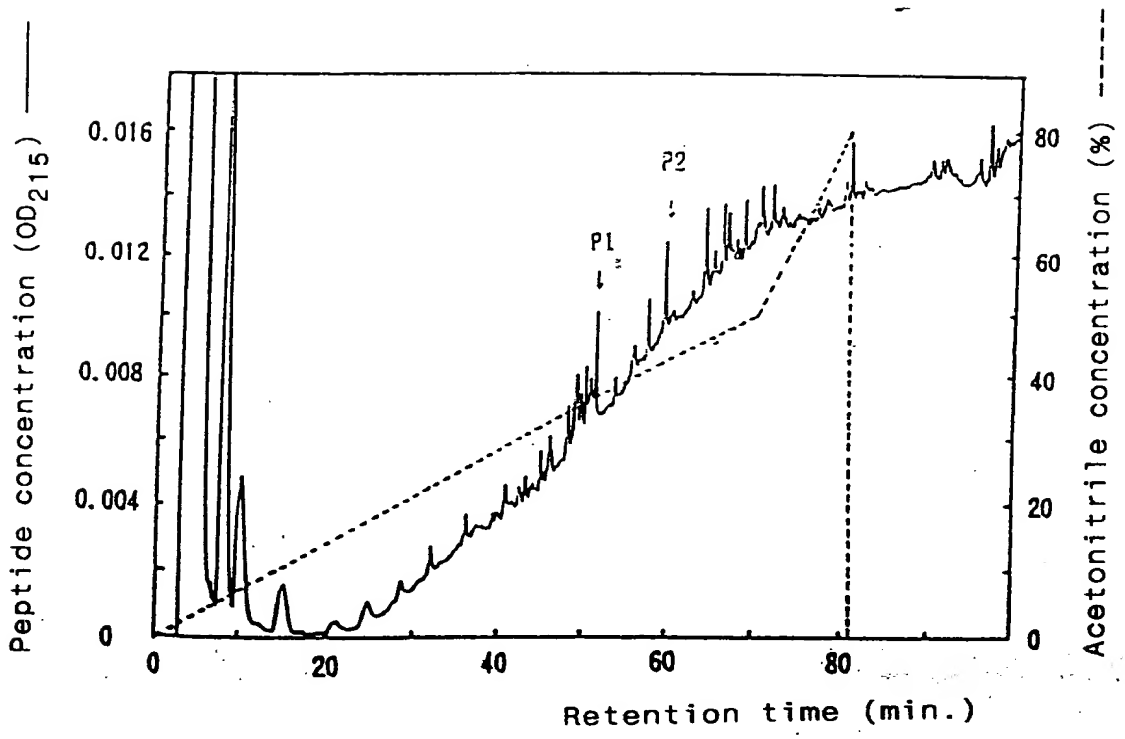
【Figure 3】


Protein concentration (OD_{215}) ——
Acetonitrile concentration (%) - - - -
0.08
0.06
0.04
0.02
0
80
60
40
20
0
0
20
40
60
80
Retention time (min.)
1
2
3
4
5
6
7
8
9

[Figure 4]


Lane
1 2 3
(k D)
94
67
43
30
20.1
14.4
120KD
60KD
Non-reduced condition
Lane
4 5 6
(k D)
94
67
43
30
20.1
14.4
Reduced condition

[Figure 5]


Peptide concentration (OD_{215}) ——
Acetonitrile concentration (%) - - - -
0.016
0.012
0.008
0.004
0
80
60
40
20
0
P1
P2
0
20
40
60
80
Retention time (min.)

【File name】 Abstract

【Abstract】

【Construction】

A novel protein having inhibitory activity on osteoclast differentiation and/or maturation.

(a) molecular weights on SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

; approximately 60 kD under reducing conditions

; approximately 60 kD and 120 kD under non-reducing conditions

(b) affinity; affinity to cation-exchanger, Cibacron blue gel and heparin column

(c) thermostability

; its biological activity to inhibit osteoclast differentiation and/or maturation activity is decreased by heating at 70℃ for 10 min. or at 56℃ for 30 min.

; its activity is lost by heating at 90℃ for 10 min.

(d) amino acid sequence

; internal amino acid sequences provided in sequence Nos. 1 and 2.

A method of producing the protein having inhibitory activity for osteoclast differentiation and/or maturation with application of cultured solution of human fibroblast cells to purification of the cultured solution by repeated adsorp-

tion and elution in ion-exchange column, heparin-column, Cibacron blue-column, and reversed phase-column chromatography.

【Effect】 The protein is useful for the treatment of bone mass reducing diseases such as osteoporosis or a biochemical test reagent.

【Selected figures】 No

[Title of the Document] DATA CORRECTION EX OFFICIO
[Document to be Corrected] Patent Application

<Recognized Information and Additional Information>

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